HIGH EFFICACY ANTISENSE RI$_\alpha$ PKA POLY-DNP OLIGORIBONUCLEOTIDES

This application claims priority to U.S. Provisional Application No. 60/431,594,

5   filed on December 5, 2003, the disclosure of which is incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates generally to antisense oligonucleotides and more particularly to antisense poly-DNP oligoribonucleotides.

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BACKGROUND OF THE INVENTION

Antisense oligonucleotides are oligonucleotides or analogs, whose sequence is complementary to a predetermined segment of mRNA. Typically, sequences of the antisense oligonucleotides are chosen so as to be complementary to a critical sequence in

15   a gene so that if the gene, or mRNA transcribed therefrom, is hybridized to the complementary antisense sequence, the gene cannot be expressed or is subjected to enzymatic degradation (see U.S. patent no. 6,291,438, incorporated herein by reference).

The use of antisense oligonucleotides has been proposed for a variety of infections as well as proliferative disorders. In view of their sequence specificity, antisense

20   oligonucleotides should be ideal anti-infective agents (Zamecnik and Stephenson, 1978; Summerton, 1979). However, their successful application in therapeutics has been delayed by problems of efficacy and drug delivery. Native oligonucleotides are not biomembrane-permeable. If delivered into cells with the help of amphipathic cations or liposomes, they could still be hydrolyzed by endogenous nucleases before reaching the

25   intended targets. In order to improve bioavailability, various types of chemically modified antisense oligonucleotides have been made by solid-state synthesis and tested with different degrees of success (Summerton and Weller, 1997; Wang et al., 1999; Nesterova and Cho-Chung, 2000; Stein, 1997). In this regard, phosphorothioate oligodeoxynucleotides (PS-oligo DNA) are considered to be more resistant to nuclease

30   digestion than corresponding phosphodiester oligodeoxynucleotides. Further, 2'-O-methyloligoribonucleotide phosphorothioates (PS-2'-O-methyloligo RNA) are considered to be more resistant to nucleases than PS-oligo DNA and can form duplexes with RNA

with higher affinity. In addition, while PS-oligo RNA/RNA duplexes are not a substrate for RNase H, PS-oligo DNA/RNA duplexes are.

Solid-state synthesis of chemically modified RNA is expensive and could also lead to stereochemical complications. For example, in a solid-state synthesized 21-nt phosphorothioate, each chiral P-atom can be in P_S or P_R configuration so that the product is really a mixture of 2^{20} isomers. This stereochemical heterogeneity could give rise to non-sequence-specific toxicity.

One promising type of oligonucleotide platform is poly-2'-O-(2,4-dinitrophenyl)-oligoribonucleotide (poly-DNP-RNA) which can be synthesized by in vitro transcription with native rNTPs followed by a single step derivatization reaction. The product has no chiral P-atoms and hence is stereochemically homogeneous. It was found that poly-DNP-RNA with DNP/nucleotide molar ratio of 0.65 to 0.75 can rapidly and spontaneously cross viral envelopes (Ashun et al., 1996). It can also slowly and spontaneously cross mammalian cell membranes without transfection reagents (Ru et al., 1999). Poly-DNP-RNAs are also resistant to degradation by ribonucleases (Rahman et al., 1996; Wang, 1996).

Several antisense poly-DNP-RNAs have been synthesized and found to inhibit viral replication and cancer growth in a sequence-specific and concentration-dependent way with no non-sequence-specific toxicity in the effective concentration range (Xin and Wang, 1998; Ru et al., 1998; Ru et al., 1999). In situ hybridization experiments showed that after staying inside cancer cells for 72 h at 37°C, an antisense poly-DNP-RNA was structurally still sufficiently intact to hybridize with a biotin-labeled sense DNA probe (Ru et al., 1999). A successful in vivo application of poly-DNP-RNA has been reported in the treatment of murine leukemia. It was observed that either i.p. or oral administration of antisense poly-DNP-RNA to MMLV-infected mice eliminated not only viremia but also the DNA of the integrated viral genome in bone marrow (Wang and Wang, 1999). The observed elimination of integrated viral genome in bone marrow by oral administration of antisense poly-DNP-RNA highlighted the bioavailability of these agents. Apparently a sufficient number of the inhibitor molecules had passed through all the membrane barriers between the alimentary canal and bone marrow and reached their target in bone marrow to trigger the elimination of the infected cells, both active and resting. The infected mice that had not been treated, or those that were treated with poly-

2

DNP-RNA of a wrong sequence all died, those that had been treated with antisense poly-DNP-RNA continued to live in apparent good health.

A common use of antisense oligonucleotides is in the field of cell proliferation disorders. An example of a gene considered to be important in cell growth regulation is

5   the RI_α/PKA gene. It is considered that RI_α is an ontogenic growth-inducing protein, and its constitutive expression disrupts normal growth processes, resulting in proliferative disorders which can lead to malignancy. An increase in RI_α/PKA is an early response to the mitogenic effects of growth factors, such as GM-CSF in human leukemic cells and TGF-α in normal rat fibroblast, and phytohemagglutinin stimulation of resting

10   lymphocytes. Increased expression of RI has also been shown to be associated with both chemical and viral carcinogenesis and oncogene-induced cell transformation. RI is the major, or sole, R subunit of PKA detected in a variety of types of human cancer cell lines and primary tumors examined (Cho-Chung, 1997). The majority of human breast cancer and colon carcinomas examined show an enhanced expression of RI and a higher ratio of

15   PKA-I/PKA-II as compared with normal counterparts (Miller et al., 1993; Bradbury et al., 1994). Importantly, the relative overexpression of the RI_α subunit of PKA was associated with poor prognosis in patients with breast cancer. Conversely, downregulation of RI_α by site-selective cAMP analogs produces growth arrest and differentiation in a wide variety of human and rodent cancer cell lines. In addition, retroviral vector-mediated

20   overexpression of RI_α provided direct evidence that RI_α plays a role in cell proliferation by regulating cell cycle progression (Tortora et al., 1994). These studies provide evidence that RI_α plays a critical role in cell proliferation. Therefore, RI_α/PKA is an attractive target for therapeutic approaches to malignancy.

In one study, the poly-DNP-RNA which was antisense to the RI_α subunit of the

25   protein kinase A (RI_α /PKA), was used to inhibit the growth of breast cancer cells which overexpressed this gene. The antisense poly-DNP RNA was found to be effective in a concentration dependent manner. Further, intraperitoneal administration of the antisense to SCID mice with transplanted MDA-MB-231 cells was found to inhibit the growth of the xenografts in concentration dependent manner to prevent metastasis and reduce

30   mortality (Ru et al., 1999, Oncology Res., 11:505-512). This antisense oligonucleotide exhibited an IC_{50} of about 22 nM in MCF-7 cells. Accordingly, for the antisense

oligonucleotides to be useful in the clinical setting, there continues to be a need to improve the efficacy of these compounds.

<u>SUMMARY OF THE INVENTION</u>

5   The present invention provides antisense oligoribonucleotides which have complementary nucleic acid sequences that recognize and bind the RI_α/PKA gene or its mRNA resulting in the down-regulation of transcription or translation. The antisense oligoribonucleotides of the present invention are targeted to the sequence corresponding to nucleotides 110-130 of the RI_α /PKA gene.

10   The antisense oligoribonucleotides of the present invention can be used for down-regulating the expression of the RI_α subunit of the protein kinase A. In one embodiment, the antisense oligoribonucleotides can be used to inhibit the growth of cancer cells.

The antisense oligoribonucleotides of the present invention can be administered in a suitable pharmaceutical carrier to an individual to effect down-regulation of the

15   RI_α /PKA gene. In one embodiment, the antisense oligoribonucleotide is administered to an individual in which the RI_α /PKA is overexpressed. Accordingly, these compositions can be used in a wide variety of conditions for inhibiting the growth of cells in which the RI_α /PKA gene is overexpressed. In the antisense oligoribonucleotides of the present invention, one or more sugar residues are modified by the substitution of DNP at the 2'-O

20   position.

The antisense oligoribonucleotides of the present invention can also be used for diagnostic purposes to identify the overexpression of the RI_α/PKA gene.

In one embodiment, the sequence of the oligoribonucleotide is poly-DNP-5'-GGCUGCGUGCCUCCUCACUGG (antisense poly-DNP RNA-21 - SEQ ID NO:1).

25   The base sequence of poly-DNP RNA-21 is complementary to that of nt 110 to 130 in RI_α mRNA. Also disclosed herein are permissible one-base mismatches of the SEQ ID NO:1.

Other investigators had previously synthesized a mixed backbone oligonucleotide (MBO) with the 18-nt sequence 5'-<u>GCGU</u>GCCTCCTCAC<u>UGGC</u> - wherein underlining

30   indicates PS-2-O-methyl oligonucleotide while the rest is PS-oligo DNA (GEM 231; SEQ ID NO:2) and showed that it inhibited the growth of targeted cancer cells with an IC_{50} value of 100 nM (Wang et al., 1999; Nesterova and Cho-Chung, 2000). We discovered

4

that by adding GGCU at the 5'-end and deleting C at the 3'-end of this 18-nt sequence of GEM 231 and replacing its MBO backbone by a poly-DNP platform, the inhibition efficacy can be increased 20 to 100-fold. The observed IC_{50} of antisense poly-DNP RNA-21 for the growth inhibition of MCF-7 breast cancer cells was 0.05 nM, and that for the growth inhibition of A549 lung cancer cells was about 3 nM. The control 21-nt poly-DNP-RNAs of similar structure but with scrambled, sense or multiple mismatched sequences were inactive.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A and 1B are representations of the sequence and concentration dependence of the inhibition of cell growth by antisense poly-DNP-RNAs for MCF-7 human breast cancer cells (1A) and A549 human lung adenocarcinoma cells (1B). The cells were plated at a concentration of 2×10^4 per well and then treated with different concentrations of poly-DNP-RNAs in the presence of Oligofectamine™. After 1 day of incubation, the medium was removed and replaced by fresh medium in the absence of RNA and Oligofectamine™. After incubation for 7 more days, the cells were collected and counted with a Coulter counter. Data are expressed as the percentage of growth inhibition in reference to the growth of untreated control cells. The data are presented as means ± SD of four independent determinations.

Figure 2 is a representation of the effect of antisense poly-DNP-RNAs on the steady-state concentration of mRNAs determined by RT-PCR assay. The amplified cDNA bands are shown in twelve lanes on the right of the molecular weight ladder in 1.5 % agarose gel. Lanes 1-6 are amplified with actin primers. Lanes 7-12 are amplified with RI_{α}/PKA primers. Lanes 1 and 7, cells without any treatment. Lanes 2 and 8, cells treated with Oligofectamine™ only. Lanes 3 and 9, cells treated with antisense poly-DNP-RNA-21. Lanes 4 and 10, cells treated with a 5-base mismatched poly-DNP-RNA. Lanes 5 and 11, cells treated with sense poly-DNP-RNA. Lanes 6 and 12, negative control without adding cDNA to the PCR mixture.

Figure 3 is a representation of Western blot analysis of PKA RI_{α} protein expression level. Treatments were as follows: Lane 1, untreated control; Lane 2, treated with Oligofectamine™ only; Lane 3, treated with antisense poly-DNP-RNA-21; Lane 4, treated with 5-base mismatched poly-DNP-RNA; Lane 5, treated with sense poly-DNP-RNA.

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Figure 4 is a representation of Western blot analysis of sequence-specific activation of Caspase-8. Treatments were as follows: Lane 1, untreated control; Lane 2, treated with Oligofectamine™ only; Lane 3, treated with antisense poly-DNP-RNA-21; Lane 4, treated with 5-base mismatched poly-DNP-RNA; Lane 5, treated with sense poly-DNP-RNA.

Figure 5 is a representation of Western blot analysis of sequence-specific cleavage of Bid. Treatments were as follows: Lane 1, untreated control; Lane 2, treated with Oligofectamine™ only control; Lane 3, treated with antisense poly-DNP-RNA-21; Lane 4, treated with 5-base mismatched poly-DNP-RNA; Lane 5, treated with sense poly-DNP-RNA.

Figure 6 is a representation of Western blot analysis of sequence-specific cleavage of Bcl-2. Treatments were as follows: Lane 1, untreated control; Lane 2, treated with Oligofectamine™ only; Lane 3, treated with antisense poly-DNP-RNA-21; Lane 4, treated with 5-base mismatched poly-DNP-RNA; Lane 5, treated with sense poly-DNP-RNA.

Figure 7 is a representation of Western Blot analysis of RI_{α}/PKA protein expression level in A549 cells. Treatments were as follows: Lane 1, untreated control; Lane 2, treated with Oligofectamine™ only; Lane 3, treated for 48 hours with 100 nM antisense poly-DNP RNA-21; Lane 4, treated for 48 hours with 100 nM 1 base mismatch (2nd position) poly-DNP RNA; Lane 5, treated for 48 hours with 100 nM 1 base mismatch (19th position) poly-DNP RNA; Lane 6, treated for 48 hours with 100 nM 1 base mismatch (21st position) poly-DNP RNA.

Figure 8 is a representation of the effect of 48 hour treatment with 100 nM poly-DNP-RNA on the steady-state concentration of mRNA in A549 cells, determined by RT-PCR assay. The amplified cDNA bands are shown in 12 lanes to the right of the molecular weight ladder (M) in 1.5% agarose gel. Lanes 1-6, amplified with actin primers; Lanes 7-12, amplified with RI_{α}/PKA primers; Lanes 1 and 7, cells without any treatment; Lanes 2 and 8, cells treated with Oligofectamine™; Lanes 3 and 9, cells treated with antisense poly-DNP-RNA-21; Lanes 4 and 10, cells treated with 1-base mismatch poly-DNP-RNA (2nd position); Lanes 5 and 11, cells treated with 1-base mismatch poly-DNP-RNA (19th position); Lanes 6 and 11, cells treated with 1-base mismatch poly-DNP-RNA (21st position).

Figure 9 is a representation of Western Blot analysis of RI_α/PKA protein expression level in MCF-7 cells. Treatments were as follows: Lane 1, treated with 300 nM MBO-21 for 48 hours; Lane 2, treated with 600 nM MBO-21 for 48 hours; Lane 3, treated with 1200 nM MBO-21 for 48 hours; Lane 4, treated with 300 nM GEM231 for

5   48 hours; Lane 5, treated with 600 nM GEM231 for 48 hours; Lane 6, treated with 1200 nM GEM231 for 48 hours; Lane 7, treated with 20 nM poly-DNP-RNA-21 for 48 hours; Lane 8, treated with 50 nM poly-DNP-RNA-21 for 48 hours; Lane 9, treated with 100 nM poly-DNP-RNA-21 for 48 hours.

Figure 10 is a representation of the thermal denaturation of duplex

10   oligonucleotides with different backbones but identical sequences except for the replacement of U by T in DNA. Thus, in the RNA/RNA duplex, the strands have the sequence of 5'-poly-DNP-GGCUGCGUGCCUCCUCACUGG (SEQ ID NO:1) and its complement; in the DNA/RNA duplex, the strands have the sequence of SEQ ID NO:1 (DNA, wherein the U's are replaced by T's) and its complementary RNA strand; in the

15   DNP-RNA/RNA, one strand has the sequence of SEQ ID NO:1, wherein the ribose residues are modified with DNP as described in Example 1; and in MBO/RNA, the structure of the antisense strand with mixed backbone is 5-GGCUGCGTGCCTCCTCACUGG (referred to herein as MBO-21 - SEQ ID NO:3), wherein the underlined nucleosides are O-methylribonucleotides and the remaining are

20   deoxyribonucleosides, and all internuclear linkages are phosphorothioate.

Figure 11 is a representation of the growth inhibitory effects of an antisense oligonucleotide of Ru et al. (1999) which has a sequence of poly-DNP-5'-GGGCGUGCCUCCUCACUGGC (SEQ ID NO:4). Open bars represent cells at day 0 and closed bars represent cell growth at day 7. Data are shown for control,

25   Oligofectamine™ only, Oligofectamine™ with: SEQ ID NO:4 at indicated concentrations and a scrambled sequence SCA 5'-GGGAGUCGCUCCGUCCUCGC 3' (SEQ ID NO:23) at indicated concentrations.

DESCRIPTION OF THE INVENTION

30   The present invention provides antisense oligoribonucleotides which have complementary nucleic acid sequences that can recognize and bind to target regions of the RI_α /PKA gene or transcribed mRNA, resulting in the down-regulation of DNA transcription, or translation of the mRNA. The antisense oligoribonucleotides provided

7

herein inhibit the growth of cells which overexpress the RI_α /PKA gene. In general, the antisense oligoribonucleotides are considered to bind to the mRNA and inhibit the translation thereof.

      The oligoribonucleotides of the present invention include sequences which are not
5   strictly antisense i.e., these sequences may have some bases which are not complementary to the bases in the sense strand but still have enough binding affinity for RI_α /PKA mRNA to inhibit the growth of cells. Further, base modifications such as inosine in the oligoribonucleotides are contemplated to be within the scope of this invention.

      As used herein, the letters "A" or "a" refer to adenine, "G" or "g" refer to guanine;
10   "C" or "c" refer to cytosine; "T" or "t" refer to thymine; and "U" or "u" refer to uracil.

      The oligoribonucleotides of the present invention are exemplified in the following sequence 5'-GGCUGCGUGCCUCCUCACUGG (SEQ ID NO:1). One or more ribose residues in this sequence are modified at the 2'-O-position with a DNP group as shown below.

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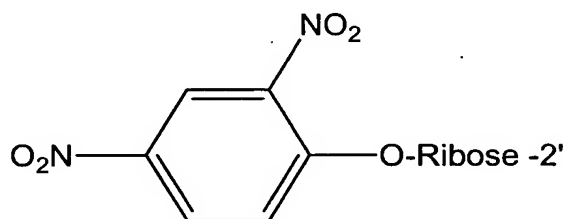
      All the internuclear linkages are phosphodiester bonds.

      Compared to the 20-mer antisense oligonucleotide of Ru et al., 1999 (5'-
20   GGGCGUGCCUCCUCACUGGC - SEQ ID NO:4), in the present invention, the first two bases, GG have been replaced with GGCU and the C at the 3' end has been deleted. Further, in comparison to the 18-mer oligonucleotide of Srivastava et al., 1999 (5'-GCGTGC<u>CUCCU</u>CACTGGC - SEQ ID NO:5), or to the GEM231 (<u>GCGU</u>GCCTCCTCAC<u>UGGC</u> - SEQ ID NO:2), in the present invention, four bases
25   have been added to the 5' end, and the C at the 3' end has been deleted. Further, it should also be noted that the oligonucleotide of Srivastava et al. and GEM231 have a mixed backbone, i.e., it is made up of both deoxyribonucleotides and ribonucleotides. Thus in the sequence of Srivastava et al. and GEM231 shown above, the underlined segment indicates PS-2-O-methyl oligoribonucleotide while the rest is PS-oligo DNA.

In general, the antisense oligoribonucleotide should have a sequence which is completely complementary to the targeted portion of the RI_α /PKA gene. However, absolute complementarity is not required, and a sequence with one mismatch is included within the scope of this invention. It is generally known that for an oligonucleotide of about 21-25 bases, a mismatch of one base can be tolerated. Data presented herein indicates that a one-base mismatch is tolerated except when the particular mismatch is at certain positions. Thus, the antisense oligoribonucleotides of the present invention include those sequences which include a mismatch at all positions except when the mismatch is a change of U to A and G to C at positions 19 and 20 respectively. While specific permissible mismatch positions have been indicated herein, one skilled in the art may readily determine the sites of other permissible mismatches from the melting point and therefore the stability of the resulting duplex. Melting points of duplexes of a given base pair composition can be determined by standard methods (see Molecular Cloning: A Laboratory Manual (J. Sambrook et al., eds)). Combination of these with cell growth inhibition data as well as protein expression and steady state mRNA data as described herein will identify the antisense oligos with permissible mismatches.

The antisense oligoribonucleotides of the present invention include sequences of from 18-30 nucleotides comprising at least the 18 contiguous nucleotides of SEQ ID NO:20 or one-base mismatch therefrom. In a preferred embodiment, the antisense oligoribonucleotides include sequences of 21-30 nucleotides comprising at least the 21 contiguous nucleotides of SEQ ID NO:1 or one-base mismatch therefrom. In another preferred embodiment, the antisense oligoribonucleotides include sequences of from 21 to 25 nucleotides. In a more preferred embodiment, the antisense oligoribonucleotides include sequences of from 21-23 nucleotides. In a still more preferred embodiment, the antisense oligoribonucleotides are 21 nucleotides long. One or more ribos residues of the nucleotides of the present invention are modified by the substitution of DNP at the 2'-O-position. In one embodiment, about 50-80% of the ribose residues are modified DNP. In another embodiment, about 65-75% of the ribose residues are modified by DNP. The ribose residue groups that are not modified by DNP can be modified by other groups. Such modifying groups are known in the art and include 2'-O-methyl RNA(OME), 2'-O-methoxy-ethyl RNA (MOE) and 2'-fluoropyrimidine RNA. It is preferable to have some free 2'-OH groups.

The antisense oligonucleotides are most advantageously prepared by utilizing any of the known chemical oligonucleotide synthesis methods. Therefore, oligonucleotides can be made by using commercially available, automated nucleic acid synthesizers. One such device, the Applied Biosystems 380B DNA Synthesizer, utilizes β-cyanoethyl

5 phosphoramidite chemistry. Further, many antisense oligonucleotides are commercially available. For example, Oligo Therapeutics, Inc. has a broad line of commercially available oligonucleotides and, further, provides contract manufacturing services for the preparation of oligonucleotides. In addition, custom oligonucleotides can be made by IDT, Coralville, IA.

10 The synthesis and derivatization of single stranded RNA (ssRNA) can be carried out as follows. ssRNA is synthesized through in vitro transcription as described before (Milligan et al., 1987) with slight modification. A template containing T7 promoter can be synthesized (such as custom synthesis by commercial sources). After synthesis of the RNA, it can be derivatized by reaction with a suitable reagent such as 1-fluoro-2,4,-

15 dinitrobenzene. The derivatized RNA is purified by the standard phenol/chloroform extraction and dialysis of the aqueous layer against water. The ratio of DNP to RNA and the actual concentration of poly DNP-RNA can be calculated from the observed absorbance at 260 and 330 nm since the oligonucleotide has absorbance only at 260 nm, whereas the DNP exhibits absorbance at both wavelengths. For larger scale synthesis, the

20 product can be separated from the reaction mixture by column adsorption and gradient elution instead of dialysis.

For administration to individuals, the antisense oligoribonucleotides of the present invention can be incorporated into convenient pharmaceutical dosage forms such as capsules, tablets, injectable, topical or inhalable preparations. Solid or liquid

25 pharmaceutical carriers can be employed. Solid carriers include, for example, starch, calcium, sulfate dehydrate, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, and stearic acid. Liquid carriers include, for example, syrup, peanut oil, olive oil, saline and water. Liposomal, viral vector, and protein conjugate preparations can also be used as carriers. Similarly, the carrier or diluent may include any

30 prolonged release material, such as glyceryl monostearate, or glyceryl distearate, alone or with wax. The amount of solid carrier varies widely but preferably, will be from about 25 mg to about 1g per dosage unit. When a liquid carrier is used, the preparation will be in the form of a syrup, elixir, emulsion, soft gelatin capsule, sterile injectable liquid such

as an ampoule, or an aqueous or nonaqueous liquid suspension. When a liquid carrier is used, it will most often be a saline solution or phosphate buffered solution. For intranasal delivery, aerosolized preparations can be used.

Pharmaceutical preparations can be made following conventional techniques of a

5   pharmaceutical chemist involving mixing, granulating and compressing, when necessary, for tablet forms, or mixing, filling, and dissolving the ingredients, as appropriate, to give the desired oral or parenteral products.

Efficacious non-toxic doses of the antisense oligoribonucleotides can be determined by clinicians having ordinary skill in the art. Typically, the dose may be

10   selected such that it results in an extracellular concentration in the vicinity of the target cells that corresponds to what has been found to be effective as shown herein. Typically, the dose may be selected from a range of 0.1 mg/kg to about 100 mg/kg, but is preferably less than 1 mg/kg. The dose can be administered to an individual, orally, rectally, by injection, or continuously. It may also be delivered to the target site, such as a tumor,

15   directly. When administered directly to the target site, a lower dose would be required.

Even in the absence of transfection agents, poly-DNP-RNAs are slowly but spontaneously transported through mammalian cell membranes (Ashun et al., 1996; Ru et al., 1999). They are also not only resistant to hydrolysis by RNases, but may actually inhibit RNases including RNase H (Rahman et al., 1996). These last two factors may also

20   contribute to the unusually high efficacy of poly-DNP-RNA-21 seen here.

The use of poly-DNP-RNA as a bioavailable platform for antisense RNAs has been demonstrated in the following publications: (1) Wang, A. and Wang, J.H. (1999) "Treatment of murine leukemia with poly-DNP-RNA", Antisense & Nucleic Acid Drug Development 9, 43-51; (2) Ru, K., Schmitt, S., James, W.I. and Wang, J.H. (1999)

25   "Antitumor effect of antisense poly-DNP-RNA in vivo", Oncology Research 11, 505-572; (3) Ru, K., Taub, M.L. and Wang, J.H. (1998) "Antisense poly-DNP RNAs as specific breast cancer inhibitor", Oncology Research 10, 389-397; (4) Xin, W. and Wang, J.H. (1998), "Treatment of duck hepatitis B by poly-DNP-RNA", Antisense & Nucleic Drug Development 8, 459-468.

30   Various types of chemically modified antisense oligonucleotides targeted at the often overexpressed RI_α gene have been successfully applied to inhibit cancer cell growth (Cho-Chung et al., 1997; Nesterova and Cho-Chung, 1995; 2000; Srivastava et al., 1999; Wang et al., 1999; Ru et al., 1998; Ru et al., 1999). The most successful example is a

11

mixed backbone oligonucleotide (MBO) with the 18-base sequence 5'-
<u>GCGU</u>GCCTCCTCAC<u>UGGC</u> (GEM231 - SEQ ID NO:2) (Wang et al., 1999). GEM231
has an IC_{50} value of 5-10 µM in MCF-7 cells and 100 nM in A549 cells. Thus for
inhibiting cancer cell growth, the present poly-DNP-RNA-21 is several orders of

5   magnitude more potent than GEM 231.

Previous studies already showed that treatment of cancer cells with chemically
modified antisense oligonucleotide can trigger apoptosis in a sequence-specific way
(Srivastava et al., 1998; Ru et al., 1998). The present work confirms that the antisense
poly-DNP-RNA-21 can kill the targeted cancer cells by silencing the RI_α gene, activating

10   caspase-8 and inducing apoptosis.

The present oligoribonucleotide can be administered to an individual including
humans in which the RI_α/PKA gene is overexpressed. As discussed in the background
section, this gene has been reported to be overexpressed in proliferative disorders such as
cancer. In the use of the oligonucletides of this invention, both single stranded as well as

15   double stranded oligoribonucleotides can be used. The oligoribonucleotides may be
administered so as to effect a reduction in the growth of cells which overexpress the
RI_α/PKA gene. The regimen may comprise one or more doses given within a short period
of time or over an extended period of time. The present antisense oligoribonucleotide
may be administered alone or in combination with other therapeutic approaches such as

20   surgical intervention, radiation, immunotherapy or chemotherapy.

Since the poly-DNP-RNA-21 of the present invention can be used to arrest or
inhibit the growth of cells which overexpress the RI_α /PKA gene, it could be used as an
anti-cancer agent with the following advantages over other chemically modified
oligoribonucleotides made by solid-state synthesis:

25   1. The synthesis of poly-DNP-RNA-21 by in vitro transcription followed by one-
             step derivatization reaction is simpler and the product has no stereochemical
             impurities.
          2. DNP-RNAs are delivered faster into mammalian cells and remain active for
             days inside the cells.
30   3. Since only extremely low dosage of poly-DNP-RNA-21 is required, sequence-
             independent non-specific toxicity should be negligible.

The antisense oligoribonucleotides of the present invention and/or complementary sequences thereof, can also be used for diagnostic purposes such as to detect the overexpression of the RI_α/PKA gene. For example, nucleic acids (mRNA or reverse transcribed DNA) can be isolated from test and control samples and hybridization

5   reactions carried out with the oligoribonucleotides provided herein. Hybridization of nucleic acid sequences are well known to those in the art and are used routinely. Thus, conditions for hybridization can be easily determined. Any increase in hybridization in the test sample over the control sample is indicative of overexpression of the RI_α/PKA.

This invention is further described in the examples provided below which are

10   intended to be illustrative and are not intended to be restrictive in any way.

EXAMPLE 1

This embodiment describes the preparation and derivatization of the antisense RNA. The templates with T_7 Promoter for in vitro transcription were customer

15   synthesized by IDT (Integrated DNA Technologies, Coralville, IA). The DNA templates used for synthesizing the antisense strand of RI_α oligo RNA were 5'-CTCAGCTGTAATACGACTCACTATAGGCTGCGTGCCTCCTCACTGG-3' (SEQ ID NO:6) and 5'-CCAGTGAGGAGGCACGCAGCCTATAGTGAGTCGTA TTACAGCTGAG-3' (SEQ ID NO:7). The method of in vitro transcription was

20   described previously (Milligan et al., 1987). Briefly, the reaction was run in 50 mM Tris Buffer (pH 8.1), 5 mM dithiothreitol, 1 mM spermidine, 0.2 µg/µl BSA, 25 mM $MgCl_2$, 5 mM NTPs, 30 µg/ml DNA template, 0.2 U/µl RNasin® Ribonuclease inhibitor (Promega, Madison, WI), 2 U/ml pyrophosphates (Sigma), 2 U/µl T_7 RNA polymerase (Promega). The reaction mixtures were incubated at 37°C for 8 h. Then 30 units RQ1 RNase-free

25   DNase (Promega) were added to the reaction mixtures and incubated at 37°C for additional 30 minutes. After digestion, the reaction mixtures were extracted with phenol and chloroform and precipitated with ethanol. The yield of the RNA product was determined by measuring optical density at 260 nm.

The RNA was derivatized as described previously (Kang and Wang, 1994) with

30   some modification. RNA (4 mg) was dissolved in 1800 µl of water and mixed with 450 µl of buffer solution (2 M $KHCO_3$, 0.1 M K_2CO_3, pH 8.8). Then 1 ml of acetone containing 160 µl of 1-fluoro-2, 4-dinitrobenzene (Sigma) was added to the reaction

mixtures. The reaction was incubated at 45°C for 5-6 hour, and the pH value was maintained around 8.8. After that, the reaction was extracted with phenol/chloroform. The resulting mixture was dialyzed against water for 3 days to remove excess 1-fluoro-2, 4-dinitrobenzene. The ratio of DNP/RNA (0.7) and the actual concentration of poly-

5   DNP-RNA were calculated from the observed absorbance at 260 and 330 nm.

EXAMPLE 2

This embodiment describes the sequence-specific inhibition of cancer cell growth by antisense poly-DNP-RNA-21. To illustrate this embodiment, experiments were

10   performed on two human cancer cell lines purchased from ATCC (Rockville, MD). MCF-7 human breast cancer cells were grown in Minimum Essential Medium (MEM) α Medium supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL, Grand Island, NY) and Insulin (5 mg/ml) (Sigma, St. Louis, MO). A549 human lung adenocarcinoma cells were grown in F-12 Nutrient Mixture (Ham) supplemented with 10% FBS (GIBCO-

15   BRL). Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

To increase the delivery of antisense poly-DNP-RNA into cells in culture, Oligofectamine™ reagent (GIBCO-BRL) was used in the poly-DNP-RNA treatment. About 2 X 10^4 cells were plated on 12 well plates 1 day before the treatment. Then RI_α/PKA antisense or control poly-DNP-RNAs were added at various concentrations in

20   the presence of Oligofectamine™ (1μl/ml). Both types of cells were incubated for 8 days with antisense poly-DNP-RNA-21, sense poly-DNP-RNA, and 5-base mismatched poly-DNP-RNA. Cells were then counted with a Coulter counter and double-checked with Trypan Blue staining. All samples were run in quadruplicate. Cells treated with Oligofectamine™ alone exhibited no cytotoxicity under any experimental conditions.

25   Figure 1 shows the concentration-dependent and sequence-specific inhibition of the growth of MCF-7 cells and that of A549 cells respectively by the antisense inhibitor poly-DNP-RNA-21. The base-sequences of the three 21-nt poly-DNP-RNA in Figure 1 are:

Antisense	5'-GGCUGCGUGCCUCCUCACUGG (SEQ ID NO:1)
Sense	5'-GGCCAGUGAGGAGGCACGCAG (SEQ ID NO:8)
Mismatched	5'-GG<u>G</u>UGC<u>C</u>UG<u>C</u>AUCC<u>A</u>CAC<u>A</u>GG (SEQ ID NO:9)

30

where each underlined base represents a mismatch. Compared to the antisense poly-DNP-RNA-21, the sense and 5-base mismatched control Poly-DNP-21 nt RNAs are essentially inactive. The IC_{50} values of poly-DNP-RNA-21 observed in MCF-7 cells and in A549 cells were 0.05 nM and 3 nM respectively which compare favorably with the

5 corresponding IC_{50} values of GEM 231 (5,000-10,000 nM in MCF-7 cells and 100 nM in A549 cells - Wang et al., 1999).

EXAMPLE 3

In this example, studies were carried out in parallel to compare the efficacy of the

10 oligoribonucleotide of the present invention with GEM231 under identical conditions. In addition, an oligonucleotide (MBO-21) which is the same sequence as the antisense oligoribonucleotide of SEQ ID NO:1, but with the mixed backbone of GEM231 was also used. Accordingly, the sequence of the MBO-21 oligonucleotide is as follows - 5' GGCUGCGTGCCTCCTCACUGG (SEQ ID NO:3) wherein the underlined segment was

15 PS-2-O-methyl oligoribonucleotide while the rest is PS-oligo DNA.

The effect of these three oligonucleotides was determined on MCF-7 cells and A549 cells under identical conditions. The results are presented in Table 1.

Table 1. IC_{50} values of oligonucleotide derivatives as specific inhibitors of growth of

20 targeted cells.

IC$_{50}$		
Oligo	MCF-7	A549
poly-DNP-RNA-21 (21-mer)	0.05 nM	3 nM
GEM231 (18-mer)	45 nM	100 nM
MBO-21 (21-mer)	30 nM	80 nM

These results confirm the comparative results in Example 2. The data shows that poly-DNP-RNA-21 (SEQ ID NO:1) is 25-fold more efficient than GEM231 in A549 cells and 900-fold more efficient than GEM231 in MCF-7 cells. The poly-DNP-RNA-21 is

25 also more efficient than MBO-21 which has the same sequence as the poly-DNP-RNA-21 but has a different backbone. Thus, it appears that both the sequence and the backbone contribute to the increased efficacy of the oligoribonucleotides of the present invention.

EXAMPLE 4

The preceding experiments (Figure 1) demonstrated that RI_α antisense poly-DNP-RNA can inhibit the growth of cancer cells in a sequence-specific manner. Since it is generally assumed that antisense inhibitors achieve cell growth inhibition through

5   suppressing the expression of specific target genes, the effect of antisense poly-DNP-RNA-21 on the steady-state concentrations of its complementary mRNA was examined by RT-PCR assay. The specific mRNAs for β-actin and RI_α/PKA were amplified by their respective primers, and the relative concentrations of the corresponding cDNAs were estimated by comparing the intensities of ethidium-stained bands in the same

10   electrophoresis gel. The experiments were carried out as follows.

MCF-7 cells (2×10^5/ well) were plated in 6-well plates one day before being treated with 10 nM poly-DNP-RNAs in the presence of Oligofectamine™. After incubation with poly-DNP-RNAs for 24 h, the cells were lysed and total RNAs were extracted using Absolutely RNA™ RT-PCR Miniprep Kit (Stratagene, La Jolla, CA).

15   The subsequent reverse transcription was carried out in the presence 2 μl Oligo $(dT)_{12\text{-}18}$ (500 μg/ml) primer, reaction buffer, 0.5 mM dNTP Mix, 10 mM DTT (GIBCO-BRL), 2 U/μl RNasin® Ribonuclease inhibitor(Promega), and 20 U/μl M-MLV reverse transcriptase (GIBCO-BRL). The reaction was run at 39°C for 1 h followed by incubation at 90°C for 10 min. About 1/20 of the mixture containing the cDNA from the

20   reverse transcription reaction was amplified with PCR in a final volume of 25 μl. The PCR reaction was performed as described with some modification. The 21 cycles of the PCR (1 min at 94°C, 1 min at 60°C, and 1 min 30 sec at 72°C) were preceded by 4 min of denaturation at 94°C and followed by 5 min of elongation at 72°C. The PCR reaction mixture has a composition of 1X reaction buffer (20 mM Tris-HCL (pH 8.4), 50 mM

25   KCl), 200 μM each of dNTPs, 1.5 mM $MgCl_2$, 0.2 μM each of the primers and 2.5 U Platinum® Taq DNA polymerase (GIBCO-BRL).

As shown in Figure 2, treatments of MCF-7 cells with antisense poly-DNP-RNA-21 did not change the mRNA level of β-actin, which was used as the internal standard, but decreased the steady-state concentration of RI_α mRNA to an undetectable level. In

30   contrast, sense or 5-base mismatched poly-DNP-RNA had no significant effect on the steady-state concentration of RI_α mRNA.

EXAMPLE 5

The sequence-specific antisense inhibition of cell growth was further confirmed by measuring the effect of the antisense poly-DNP-RNA-21 on the expression of the target gene products (RI_α protein) by Western blotting analysis. Cell extracts were

5   prepared as follows. Cells (2 X 10^5/ well) were plated in 6-well plates one day before treatment with 10 nM poly-DNP-RNAs in the presence of Oligofectamine[TM]. After incubation of MCF-7 cells with poly-DNP-RNAs for 24 h, cells were washed once with PBS and lysed with 200 μl boiling lysis buffer (1% SDS, 1.0 mM sodium ortho-vanadate, 10 mM Tris pH 7.4). Protein concentration was determined by BCA Protein Assay

10   Reagent Kit (Pierce, Rockford, IL), using bovine serum albumin (BSA) as a standard.

Following preparation of cell extracts, total protein (20 μg) was run on NuPAGE® 4-12% Bis-Tris Gel with MOPS running buffer and then transferred to InvitrolonTM PVDF membranes (Invitrogen, Carlsbad, CA). Blotted membranes were developed using WesternBreeze® Novex Chromogenic Western Blot Immunodetection

15   Kit (Invitrogen). Monoclonal antibodies against RI_α, Bid, Bcl-2, and Caspase-8 were purchased from BD Biosciences (San Diego, CA).

Figure 3 illustrates the effect on RI_α protein expression of different poly-DNP-RNAs at same concentration. In antisense poly-DNP-RNA-21 treated MCF-7 cells, the expression level of RI_α protein was barely detectable, whereas in the sense and 5-base

20   mismatched poly-DNP-RNA treated cells, the expression levels of RI_α protein were comparable to those in the untreated control cells.

EXAMPLE 6

A previous study showed that inhibition of growth of cancer cells by antisense

25   poly-DNP-RNAs results in apoptosis (Ru et al., 1998). This example describes the effect of an antisense oligoribonucleotide of the present invention on the status of proteins related to the apoptosis pathway. The molecular events involved in the activation of apoptosis signal transduction pathway was studied by Western blotting analysis. Cells were treated with poly-DNP RNAs and processed for Western blotting as described in

30   Example 5.

As shown in Figure 4, when MCF-7 cells had been treated with antisense poly-DNP-RNA for 24 h, activation of caspase-8 was indicated by the disappearance of the

procaspase band and the formation of two smaller activated subunits of caspase-8. The activation of caspase-8 was sequence-specific because when MCF-7 cells were treated with either sense or 5-base mismatched poly-DNP-RNA, the procaspase-8 still remained intact after 24 h incubation. In addition, as shown in Figure 5, Bid also depleted in a

5   sequence specific manner.

Among the numerous factors known to modulate the apoptosis signal transduction pathway, caspase-8 is the key initiator caspase in the death-receptor pathway (Hengartner, 2000). Caspase-8 is produced as a proenzyme (55/50 kDa doublet) which upon receptor aggregation is proteolytically cleaved into smaller subunits of 40/36 (doublet), and 23

10   kDa (Boesen-de Cock et al., 1998; 1999). Cross-talk and integration between the death receptor and mitochondrial pathways is provided by Bid, a pro-apoptotic Bcl-2 family member. Caspase-8 mediated cleavage of Bid greatly increases its pro-death activity, and results in its translocation to mitochondria, where it promotes cytochrome c exit (Hengartner, 2000). The observed effects on Bid are consistent with its role in apoptosis.

15

EXAMPLE 7

This example describes the effect of treatment with an antisense oligoribonucleotide of the present invention on the status of Bcl-2 protein. High expression of Bcl-2 has been shown in human breast cancer cell lines (Haldar et al., 1994).

20   As an anti-apoptotic factor, Bcl-2 protein blocks apoptotic stimuli such as growth factor deprivation, radiation, heat-shock, virus and most of the chemotherapeutic agents (Hockenbery et al., 1990; Reed, 1995). As shown in Fig. 6, in MCF-7 cells treated with antisense poly-DNP-RNA-21, the expression level of Bcl-2 protein had been reduced significantly compared to those treated with sense and 5-base mismatched poly-DNP-

25   RNA.

EXAMPLE 8

This embodiment demonstrates that a one-base mismatch is permissible for the antisense oligoribonucleotides of the present invention. For this example, the bases in

30   SEQ ID NO:1 were replaced at indicated positions as shown in the Table 3 and IC_{50} was determined for A549 cells. As shown in Table 2, the antisense oligoribonucleotide without any replacements has an IC_{50} of about 3 nM. When single replacements were made at positions up to 18 and then at position 21, the oligoribonucleotide was still

effective at comparable concentrations. However, when a change was made at positions 19 and 20, as indicated in Table 2, the oligoribonucleotides were found to be inactive.

Table 2

Positional Dependence of the Effect of Single Mismatch on the Inhibition of AS-RI$_\alpha$

Poly-DNP-RNA in A549 Cells

Position of Mismatch	Mutation	SEQ ID NO:	IC50 (nM)	Inhibition Efficacy
none	none	1	3.0	+
21	G→ C	10	12	+
20	G→ C	11	inactive	-
19	U→ A	12	inactive	-
18	C→ G	13	20	+
17	A→ U	14	9	+
16	C→ G	15	8	+
15	U→ A	16	7.5	+
14	C→ A	17	5.6	+
11	C→ A	18	4.5	+
2	G→ C	19	3.5	+

To confirm that changes of U to A and G to C at positions 19 and 20 respectively, were not permissible while mismatches at other positions were permissible, Western blot analysis of the RI$_\alpha$/PKA protein was carried out in A549 cells incubated with mismatches at positions 2, 19 and 21. The results are shown in Figure 7. These results indicate that untreated controls expressed RI$_\alpha$/PKA (Lane 1) and treatment with the transfecting agent only, did not decrease this expression (Lane 2). However, when these cells were treated with the antisense poly-DNP-RNA-21 (SEQ ID NO:1), a significant decrease in the expression of RI$_\alpha$/PKA was observed (Lane 3). A reduction was also observed for the mismatch at position 2 (Lane 4) and position 21 (Lane 6). However, no reduction was observed when the mismatch was at position 19 (Lane 5).

In another experiment, the effect of some of the one-base mismatches was determined on the steady-state levels of mRNA for RI$_\alpha$/PKA. A549 cells were incubated

with one-base mismatches at positions 2, 19 and 21 and RT-PCR was carried out.
Primers used for actin were 5'-GGGATAGCACAGCCTGGATAGCA-3' (SEQ ID
NO:24) and 5'-GATGATATCGCCGCGCTCGTCGTC-3'(SEQ ID NO:25) and primers
used for RI_α/PKA were 5'-GCTAAAGCGGCCATTGTCTTGT-3' (SEQ ID NO:26) and

5   5'-AGTACCGCCGCCAGTGAGGAGG-3' (SEQ ID NO:27). The results are shown in
Figure 8. The results indicate that control cells showed RI_α/PKA (Lane 7) which was not
affected by the transfecting agent alone (Lane 8). However, the poly-DNP RNA of SEQ
ID NO:1, reduced the concentration of the RI_α/PKA mRNA (Lane 9). A similar effect
was observed for the one-base mismatch at position 2 (Lane 10). The one-base mismatch

10   at position 21 also appears to reduce the level (Lane 12). However, the one-base
mismatch at position 19 (Lane 11) did not reduce the level as compared to controls. This
experiment further confirms that one-base mismatches are permissible except when U at
position 19 is changed to A and G at position 20 is changed to C. Based on the data and
description provided herein other permissible mismatches at various positions can be

15   determined by those skilled in the art.

EXAMPLE 9

In this embodiment, the efficacy of the antisense poly-DNP- RNA-21 of the
present invention was compared to the mixed backbone sequence of MBO-21 (SEQ ID

20   NO:3) and to GEM231 (SEQ ID NO:2) with respect to the effect on the expression of
RI_α/PKA. To carry out this experiment, MCF-7 cells were incubated with the antisense
poly-DNP RNA-21, MBO-21 and GEM231. After various times, the expression of
RI_α/PKA was evaluated by Western blotting. The results are shown in Figure 9. The
results show that no significant reduction in the expression of RI_α/PKA was observed

25   with MBO-21 and GEM231 even up to concentrations of 1.2 μM while a significant
effect was observed with 50 and 100 nM for the poly DNP oligoribonucleotide of the
present invention. These data indicate that gene silencing efficacy of the poly-DNP-
RNA-21 is several folds higher than either the MBO-21 or the GEM231.

30   ## EXAMPLE 10

This embodiment describes the growth inhibiting ability of antisense
oligoribonucleotides of varying lengths. For this experiment, antisense
oligoribonucleotides of 18, 20, 21 and 23 bases were synthesized and derivatized with

20

DNP as described in Example 1. The sequence of the 21-mer used in this experiment is the same as SEQ ID NO:1. For the 18-mer, the three 3' end nucleotides, UGG, were deleted and for the 20-mer, the last 3' end nucleotide G was deleted. For the 23 mer, two nucleotides, CG were added to the 3' end to provide the sequence poly-DNP-5'-

5   GGCUGCGUGCCUCCUCACUGGCG (SEQ ID NO:22). Cell growth assays were carried out as described in Example 2 on A549 and MCF-7 cells. The results are shown in Table 3 below.

Table 3. Values for cell growth inhibition by RI_α/PKA targeting antisense DNP-RNA of
10   different lengths.

Length	Sequence	SEQ ID NO:	MCF-7
18	5'-GGCUGCGUGCCUCCUCAC	20	~ 20-30 nM
20	5'-GGCUGCGUGCCUCCUCACUG	21	~ 20-30 nM
21	5'-GGCUGCGUGCCUCCUCACUGG	1	0.05 nM
23	5'-GGCUGCGUGCCUCCUCACUGGCG	22	0.8 nM

These results indicate that truncating the 3' end of SEQ ID NO:1 results in an oligoribonucleotide which is active but not to the same extent as poly-DNP-RNA-21.
15   However, extending the 3' end results in an antisense oligoribonucleotide which is comparable to the poly-DNP-RNA-21 for inhibiting the growth of cells.

EXAMPLE 11

This example describes the thermal denaturation of duplex oligonucleotides. The
20   thermal denaturation of duplex oligonucleotides was monitored by UV-absorption at 260 nm. For comparison the denaturation curves of four duplex oligonucleotides with different backbones but identical sequence of bases, except for the replacement of U by T in DNA are shown in Figure 10.

Among the four duplexes examined in Figure 10, poly-DNP-RNA/RNA has the
25   highest hybridization affinity. The observation that the transition temperature of poly-DNP-RNA/RNA is even higher than that of native RNA/RNA (siRNA) indicates that the DNP-groups not only do not interfere with base-pairing but may aid hybridization via

weaker interactions. These observations also suggest that during the derivatization of RNA by the present procedure, the bases themselves are not modified with DNP.

EXAMPLE 12

5   This embodiment describes the use of the antisense oligoribonucleotides of the present invention to arrest or reduce the growth of malignant cells. The antisense oligoribonucleotides can be administered to an individual who has been diagnosed with cancer in which the cells overexpress this gene. A detailed description of various methods that are known in the art for therapeutic applications of antisense

10   oligoribonucleotides have been provided.

For this embodiment, human cancer xenograft models can be established by standard methods (see Wang et al., 1999). Briefly, SCID mice can be commercially obtained. Cells (such as A549 and MDA-MB231) can be cultured and harvested in a suitable medium (such as Ham's F-12 optionally containing basement membrane matrix),

15   and injected or implanted into the mice. The cells can be monitored by general clinical observation, determination of body weight and tumor weight. Tumor growth can be recorded and tumor mass calculated from the two perpendicular diameters of the implant. For determination of the effect of poly-DNP RNAs, the oligoribonucleotides are dissolved in a suitable carrier such as physiological saline and administered (such as i.p.).

20   The volume, dose and frequency of oligos injected can be varied. Control animals can be administered the sense oligoribonucleotide or scrambled sequence oligoribonucleotide. Tumor growth of the xenografts, and other parameters such as expression of RI_{α}/PKA can be studied in the mice and compared to controls.

In this regard, it is also important to note that an antisense oligonucleotide of 20

25   nucleotides in length (SEQ ID NO:4), which has 17 nucleotides common with the present nucleotide, was shown to be effective in vivo in SCID mice in which MDA-MB-231 cells had been implanted (Ru et al., 1999). The present antisense oligoribonucleotide is of a comparable length and nucleotides 5-21 of the present antisense oligoribonucleotide poly-DNP-RNA-21 are identical to the 20-mer of Ru et al. oligonucleotide. To compare the

30   efficacy of the antisense oligonucleotide of Ru et al., MCF-7 cells were treated with poly-DNP-RNA of SEQ ID NO:4 and a scrambled sequence 5'-GGGAGUCGCUCCGUCCUCGC 3' (SEQ ID NO:23). The results are shown in Figure 11. The IC_{50} for the antisense oligonucleotide of SEQ ID NO:4 is about 22 nM. These

results show that the present antisense oligonucleotide is several folds more efficacious than SEQ ID NO:4. Since SEQ ID NO:4 has been demonstrated to have in vivo effects, the present antisense oligoribonucleotide is expected to be more efficacious than SEQ ID NO:4 in vivo also.

5   Another oligonucleotide, GEM231, a mixed backbone oligo targeting the RI$_\alpha$/PKA gene has been shown to be effective in vivo and is currently undergoing clinical trials (Wang et al., 1999). As demonstrated herein the antisense oligoribonucleotide of the present invention is more efficacious than either the 20 mer of Ru et al. or the 18-mer mixed backbone GEM231. Thus, it is expected that similar administration of the
10   antisense oligoribonucleotide of the present invention in vivo will provide for an efficacious arrest or inhibition of growth of cancer cells.

       While specific embodiments have been presented herein, routine modifications will be apparent to those skilled in the art and are intended to be within the scope of the
15   invention.

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